

Ahmed, Salman E., Rehman, Shafiq, Edilbe, M., Jonker, Leon and Canelo, Ruben (2017) Can neutrophil-lymphocyte ratio predict operators' difficulty in early cholecystectomies; a retrospective cohort study. *Annals of Emergency Surgery*, 2 (3). art. 1016.

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Research Article

Can Neutrophil-Lymphocyte Ratio Predict Operators' Difficulty in Early Cholecystectomies; A Retrospective Cohort Study

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Submitted: 22 June 2017

Accepted: 30 June 2017

Published: 30 June 2017

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ISSN: 2573-1017

OPEN ACCESS

Keywords

• Gallbladder; Intra operative difficulty; Cholecystitis; Difficult cholecystectomy; early cholecystectomy; neutrophil-lymphocyte ratio; predictive value

Abstract

Background: Recent years has seen an increased trend toward “early cholecystectomy” following acute cholecystitis. Operators whilst performing cholecystectomy during acute cholecystitis commonly finds varying degree of inflammation ranging from soft omental adhesions to densely adherent gangrenous gall bladder, which is not only associated with morbidity but is often technically challenging. In this study we aim to retrospectively evaluate the role of neutrophil-lymphocyte ratio (NLR) and its association with operative difficulty and length of stay for patients who underwent ‘hot cholecystectomy’.

Methods: An anonymised retrospective single-centre cohort study using operative notes and clinical data. Chi-squared test and Mann-Whitney U-test were applied to determine significance between variables in the predetermined low NLR (<5) and high NLR (>5) groups. Multiple linear regression was applied for assessing any significant relationships between NLR and operative difficulty, length of stay (LOS) and post-operative stay (POS).

Results: Patients with a high NLR > 5 level upon admission are on average older (median 44 vs 60 years; p-value 0.003), have a longer hospital stay (median 4 vs 5 days; 0.005), their operations takes longer (median 81 vs. 98 minutes; p=0.026), and operations are deemed more difficult (13% vs. 33%; p=0.035) as judged by surgeons and evident by intra operative parameters. In linear regression increased age was found to be associated with higher NLR (p=0.035). Presence of gangrene was significantly associated with both LOS and POS in regression analysis (p-value 0.044 and 0.015 respectively).

Conclusions: In performing an ‘early cholecystectomy’ a higher NLR on admission is associated with operators’ difficulty. However, a NLR cut off of 5 is not significantly linked to increased length of stay. Nonetheless, NLR on admission can be applied to stratify acute cholecystitis to plan surgery and anticipate difficult operation. NLR when combined with age can be utilised as guide to prioritise the urgency of operation and as prognostic predictor of possible post op complications.

INTRODUCTION

Acute cholecystitis is one of the common surgical emergencies. Since its introduction, laparoscopic cholecystectomy has become the preferred operative choice to remove the gall bladder. In recent years there has been a shift towards ‘early cholecystectomy’ i.e., removing the gall bladder during an acute attack of cholecystitis. It is strongly recommended by national institute of clinical excellence (NICE) to offer cholecystectomy within one week of acute cholecystitis [1]. Several clinical, radiological and haematological predictors of severity of inflammation are in use, including systemic inflammatory response, ultrasound scan, white cell count, C reactive protein (CRP), platelet lymphocyte ratio and others [2-6]. Systemic inflammatory response syndrome SIRS combined with clinical assessment plays a significant role in assessment of patient with any inflammatory or infective / septic condition e.g. cholecystitis. Raised inflammatory markers such as white blood cell and

CRP although are very sensitive in suggesting the presence of inflammation / infection but, cannot suggest clinical severity of condition. Investigators have found a relative lymphocytopenia in variety of infective and septic conditions, its relation to the disease severity has been investigated [7-10]. In an attempt to understand the immune response the neutrophil lymphocyte ratio (NLR) has been investigated in variety of inflammatory / infective and tumoral conditions [7-13].

As an indicator of severity of inflammation, the prognostic value of NLR in different conditions such as acute cholecystitis, acute appendicitis, and sepsis due to multiple causes requiring intensive care, has been studied [7-10,14]. Investigators have also found a higher level of plasma cytokines (IL-1ra, IL-6, IL-7, IL-8, IL-12) in patients with high NLR [13,15,16]. In this study we aim to utilise NLR as surrogate indicator of up-regulation of immune system which in turn translates in to operators’ difficulty

whilst doing an 'early cholecystectomy'. The association of NLR with length of stay was also analysed.

Definition of difficult gall bladder and cut off value for neutrophil-lymphocyte ratio NLR

Strict criteria of difficult operation was applied, difficult operation was defined as "an operation involving inflammation, empyema, adhesions, gangrene or perforation of gall bladder leading to operator's difficulty in dissecting the Calot's triangle with or without subtotal operation" [17-19]. All the intra operative findings attributed to difficult gall bladder were analyzed in addition to surgeon's judgment about operative difficulty (as described in operating notes).

Lee et al. [7], described an NLR of 3 as cut off point to differentiate between and simple and severe cholecystitis. Beliaev et al. [10], have demonstrated the cut off value to be diagnostic at 3.25 and 4.17 between mild and moderate-severe cholecystitis respectively. Kelly et al. [8], described the value of $NLR > 6.35$ to be associated with severe appendicitis. Sharaiha et al. [12], and Carruthers et al. [12], separately described $NLR > 5$ as having prognostically predictive value in two separate tumoral processes. Patel et al. [20], describes $NLR > 11$ significantly associated with length of stay in acute cholecystitis. While studying patients admitted to intensive care due to infection Liu et al. [9], found NLR as indicator of disease severity, at value of 11.11, 22.67 and 31.50 associated with sepsis, severe sepsis and septic shock respectively. An agreed cut off $NLR > 5$ was used to divide patient in two groups to observe how the intensity of immune response based on disease severity translates in to a difficult operation and associated morbidity.

METHODS

Study design & subjects

We retrospectively reviewed clinical data of early cholecystectomies performed in a busy district general hospital between October 2014 to September 2015. During this period, 89 patients underwent emergency laparoscopic cholecystectomies. Inclusion criteria were: emergency hospital admission; hot gallbladder confirmed through radiological diagnostic (USS 77% $n=62$ and CT scan 33% $n=19$) test pre-operatively; operated within same admission episode. Exclusion criteria were: non-inflamed emergency cholecystectomy (e.g. gallstone pancreatitis), patient requiring CBD stone extraction before cholecystectomy (Figure 1). After applying inclusion and exclusion criteria, 81 cases were analysed as part of this retrospective cohort study. Acute cholecystitis was defined using the 2007 Tokyo classification [16].

NLR was calculated from results of blood tests taken on the day of admission. A pre-analysis agreed cut-off value of 5 was used to divide patients into two groups, $NLR < 5$ and $NLR > 5$, for sub analysis. The operating and medical notes for all the patients were used to collect data. Each operation was done by a senior grade surgeon (registrar level and above with consultant scrubbed) according to departmental policy. Surgeon's observation about difficulty of procedure was also taken into account for analysis.

Statistical analysis

Data was collated in Microsoft Excel 2007, and further analyzed and interpreted using Excel and SPSS Statistical

Inclusion criteria	Exclusion criteria
<ul style="list-style-type: none"> Emergency admission Clinical signs suggesting Cholecystitis (Tokyo classification) Radiological confirmation Operation during same admission 	<ul style="list-style-type: none"> Emergency operation done for non inflamed gall bladder i.e., Pancreatitis Cholecystitis with CBD stones requiring ERCP. Requiring Intervention radiology due to poor physiologic status

Figure 1 Inclusion and Exclusion criteria.

Package for Social Sciences (SPSS) version 17.0 (SPSS Inc. Chicago, IL, USA, 2007). Data for demographic variables, such as age and gender were collected, as well as clinical outcomes including the presence of any gangrene or pus, intra-operative drain insertion in gall bladder fossa, incomplete operation, conversion to open surgery. Post-operative complications based on Clavien-Dindo classification were also assessed. Differences for parameters between different NLR subgroups were assessed through Chi-squared tests and Mann-Whitney U-test as indicated. Multiple linear regression analysis was applied to investigate any parameters that may be significantly associated with longer overall length of stay, and /or longer post-operative length of stay in hospital.

RESULTS

$NLR < 5$ group had 32 patients and $NLR > 5$ had 49 patients. As expected female were more in both groups. Median age was found to be 60 years in $NLR > 5$ group as compared to 43 years in other group ($p=0.003$). There was difference of one day in length of stay noticed in both groups ($p=0.005$) (Table 1).

Intra operative details were analyzed and we found significant difference in operative time 81 mins vs. 98 mins in two groups ($p=0.026$). Operators' difficulty as judged by surgeon was also found to be significant at 33% in $NLR > 5$ group comparing 13% in $NLR < 5$ group ($p=0.035$). In addition it was found, there were higher incidence of empyema, gangrene and adhesions in $NLR > 5$ group (Table 2). Also higher rates of conversion to open and intra operative drain insertion were seen in $NLR > 5$ group (Table 3). *Amongst the operations judged difficult 9% vs. 16% in respective groups were partial or subtotal cholecystectomy (partial means leaving posterior wall attached but cystic duct successfully clipped, subtotal means safe clipping of cystic duct not performed gall bladder tied or sutured at the level of hartmann's pouch).*

In multiple linear regression analysis it was found that the age is a significant factor in $NLR > 5$ group ($p=0.036$). No association of $NLR > 5$ was seen with either overall length of stay or post-operative stay (Table 3).

We performed further multiple linear analyses on length of stay and post operative stay in both groups. It was found that the overall length of stay and post operative stay was associated independently with presence of gangrenous gall bladder in both the groups ($p=0.044$ and $p=0.015$ respectively) (Table 4). It is important to mention no mortality in the study population with only 1 patient requiring level 3 care for respiratory complications later discharged.

DISCUSSION

It is estimated around 66,660 cholecystectomies are performed each year in UK out of which 61,220 are performed laparoscopic [21]. Overall annual cost to the NHS for performing

Table 1: Overview of variable statistics for groups NLR < 5 and NLR > 5.

Variable	NLR < 5 (n= 32)	NLR > 5 (n= 49)	p-value
Gender (male / female)	22% / 78% (7 / 25)	39% / 61% (19 / 30)	0.14 ^A
Age (yrs, median)	43	60	0.003 ^{B,#}
Length of Stay LOS (days, median)	4	5	0.005 ^{B,#}
Post-op stay POS (days, median)	1.5	2	0.135 ^B
ASA grade (median)	1.5	2	0.63 ^A

[#]Statistically significant at p-value < 0.05; ^AChi-squared test; ^BMann-Whitney U-test.

Table 2: intra operative variable statistics for groups NLR < 5 and NLR > 5.

INTRA OPERATIVE FINDINGS			
Variable	NLR < 5 (n= 32)	NLR > 5 (n= 49)	p-value
Operation time (min, median)	81	98	0.026 ^{B,#}
Gangrene present	3% (n=1)	14% (n=7)	0.094 ^A
Pus present	19% (n=6)	27% (n=13)	0.39 ^A
Omental adhesions present	44% (n=14)	59% (n=29)	0.20 ^A
Drain required	28% (n=9)	51% (n=25)	0.052 ^A
Conversion required	0% (n=0)	10% (n=5)	0.059 ^A
Operation completed	91% (n=29)	84% (n=41)	0.35 ^A
Operation difficulty, judged 'yes' by surgeon	13% (n=4) (9% n=3 parial or sub-total) ²	33% (16) (16% n=8, partial or sub-total) ²	0.035 ^{A,#}

[#]Statistically significant at p-value < 0.05; ^AChi-squared test; ^BMann-Whitney U-test.

Table 3: Multiple linear regression analysis for categorized NLR (<5 and >5).

Outcome measure		
Variable	Standardized coefficient Beta	p-value
Gender	-0.015	0.91
Age	0.29	0.036 [#]
ASA level	-0.094	0.46
Operation time	0.18	0.20
Inflamed	-0.12	0.36
Gangrenous	0.15	0.36
Pus present	-0.003	0.99
Omental adhesions	0.039	0.77
Operation difficulty	0.052	0.80
Operation completed	0.029	0.85
Drain use	-0.035	0.84
Conversion	0.092	0.51
LOS	0.21	0.21
POS	-0.13	0.43

[#] statistically significant, p-value <0.05

this operation is around £111.6 million [21]. NICE has given a strong recommendation to offer early laparoscopic cholecystectomy within one week of diagnosis of acute cholecystitis [1]. It has previously been shown that when performing a planned elective cholecystectomy with history of cholecystitis some patients are more of a challenge to surgeons than others. Those patient groups who initially presented as an acute cholecystitis, and subsequently underwent delayed planned elective surgery, had more conversion rates and their operation times were longer Lorenz et al. [22]. Previous attempts have been made to predict the operative difficulty during a cholecystectomy

using radiological tools [2,3].

In this study we demonstrate there is a significant difference in median age between 2 study groups. An increase age was found to be associated with high NLR. It has been described that an increased age is associated with severity of cholecystitis and prolonged length of stay by Lee et al. [7] and Patel et al., [20]. It has also been observed that an increased age with high NLR is associated with non survivor group of intensive care patients admitted with sepsis [9].

Although the NLR utility to predict and estimate the

Table 4: Multiple linear regression analysis for LOS and POS.

Outcome measure	Length of stay (LOS)		Post-operative length of stay (POS)	
	Standardized coefficient Beta	p-value	Standardized coefficient Beta	p-value
Gender	0.049	0.70	-0.0004	0.98
Age	0.24	0.064	0.21	0.11
NLR value	0.058	0.64	-0.040	0.74
ASA level	-0.059	0.62	-0.076	0.52
Operation time	-0.023	0.86	0.13	0.30
Inflamed	-0.26	0.027 [#]	-0.026	0.82
Gangrenous	0.28	0.044 [#]	0.34	0.015 [#]
Pus present	-0.20	0.12	-0.13	0.31
Omental adhesions	0.024	0.84	0.030	0.80
Operation difficulty	0.12	0.53	-0.29	0.12
Operation completed	-0.19	0.16	-0.15	0.29
Drain use	0.16	0.31	0.25	0.12
Conversion	0.14	0.26	0.22	0.082

[#] statistically significant, p-value <0.05

severity of inflammation has been described in the literature (12,23,24,25). To our knowledge this study is the first to describe the relation of high NLR with a difficult cholecystectomy (13% vs. 33%, p=0.035 Table 2). We appreciate that judging the operator's difficulty of performing an operation has inherited subjective bias, care was taken to thoroughly investigate the intra operative findings. Although 13% operation were deemed difficult in NLR < 5 group 9% (n=3) were partial or subtotal, comparing to NLR > 5 group where 33% operations were judged as difficult by surgeon but only 16% (n=8) were partial or subtotal rest of all the difficult operation were complete (Table 2). In our study group no operation was abandoned and bailed out with cholecystostomy tube.

Individual parameters of operative time, inflammation, empyema, adhesions, gangrene, intra operative drain and conversion to open were analyzed between 2 groups. Significant difference was seen in operative time (p=0.026 Table 2). In multivariate regression analysis the association was not found to be independent but, longer median operation time 98 vs. 81 min (p=0.026), the increase incidence of empyema (27% vs. 19%), gangrenous gall bladder (14% vs. 3%), adhesions (59% vs. 44%), intra operative drain insertion (51% vs. 28%) and conversion to open (10% vs. 0%) in NLR > 5 group indicate that NLR >5 can be utilized as a guide to predict and plan a difficult operation.

In this study an independent association between the NLR and length of stay at cut off value of 5 could not be established. However it was interesting observation in multivariate regression to see the association of the incidence of gangrenous gall bladder with increased length of stay more importantly with post operative stay (Table 4).

Our study was limited with its retrospective cohort. There was an element of surgeons' and experience bias however this was thought to be minimum given there was always a consultant scrubbed for a 'hot cholecystectomy'. Due to lack of agreement

at cut off value of NLR at cut off value in literature [7-10,20] the departmentally agreed cut off was utilised by the authors to divide patients in 2 groups. Moreover the overall length of stay analysis was confounded by the availability of radiological service to confirm the suspected clinical diagnosis.

After the finding of this study a prospective study in out institute is being carried out. NLR > 5 is being used as a guide to inform patients of increased likelihood of difficult operation and potential complications, but also helps the operating surgeon to plan and operate with appropriate help and expertise around.

CONCLUSION

It is therefore suggested that higher NLR > 5 can be use as a guide to predict and plan a difficult cholecystectomy. NLR being simple to calculate should be used as common practice with patients presenting with acute cholecystitis. High NLR > 5 when combined with age not only predicts the operating challenge and technical difficulty but also indicates severity of underlying immune response. Its utility may also include prioritisation, planning appropriate expertise, and as guidance to inform patients in consent process of possible complication and what to expect.

REFERENCES

1. Nice Guidelines on management of acute cholecystitis.
2. Roberto Santambrogio, Marco Montorsi, Paolo Bianchi, Enrico Opocher, Luca Schubert, Maurizio Verga, et al. Technical Difficulties and Complications during Laparoscopic Cholecystectomy: Predictive Use of Preoperative Ultrasonography. 1996; 20: 978-982.
3. Peter Corr, Jeremy JT, Lau WY, Dawson JW, Li AK. Preoperative ultrasound to predict technical difficulties and complications of laparoscopic cholecystectomy. Am J Surg. 1994; 168: 54-56.
4. Mok KW, Reddy R, Wood F, Turner P, Ward JB, Pursnani KG, et al. Is C-reactive protein a useful adjunct in selecting patients for emergency cholecystectomy by predicting severe/gangrenous cholecystitis? International journal of surgery. 2014; 12: 649-653.

5. Cagatay Aydin, Gülüm Altaca, Ibrahim Berber, Koray Tekin, Melih Kara, Izzet Titiz. Prognostic parameters for the prediction of acute gangrenous cholecystitis. *Journal of hepato-biliary-pancreatic science*. 2006; 13: 155-159.
6. Wang AJ, Tsang-En Wang, Ching-Chung Lin, Shee-Chan Lin, Shou-Chuan Shih. Clinical predictors of severe gallbladder complications in acute a calculous cholecystitis. *World J Gastroenterology*. 2003; 9: 2821-2823.
7. Lee SK, Lee SC, Park JW, Kim SJ. The utility of the preoperative neutrophil-to-lymphocyte ratio in predicting severe cholecystitis: a retrospective cohort study. *BMC surgery*. 2014; 14: 100.
8. Kelly ME, Khan A, Riaz M, Bolger JC, Bennani F, Khan W. The Utility of Neutrophil-to-Lymphocyte Ratio as a Severity Predictor of Acute Appendicitis, Length of Hospital Stay and Postoperative Complication Rates. *Dig surg*. 2015; 32: 459-463.
9. Xuan Liu, Yong Shen, Hairong Wang, Qinmin Ge, Aihua Fei, Shuming Pan. Prognostic Significance of Neutrophil-to-Lymphocyte Ratio in Patients with Sepsis: A Prospective Observational Study. *Mediators of Inflammation*. 2016 (2016), Article ID 8191254.
10. Beliaev am, Neville Angelo, Michael Booth, Colleen Bergin. Evaluation of neutrophil-to-lymphocyte ratio as a potential biomarker for acute cholecystitis. *Journal of surgical research*. 2017; 209: 93-101.
11. Kishi Y, Kopetz S, Chun YS, Palavecino M, Abdalla EK, Vauthey JN. Blood neutrophil-to-lymphocyte ratio predicts survival in patients with colorectal liver metastases treated with systemic chemotherapy. *Ann Surg Oncol*. 2009; 16: 614-622.
12. Sharaiha RZ, Halazun KJ, Mirza F, Port JL, Lee PC, Neugut AI, et al. Elevated preoperative neutrophil:lymphocyte ratio as a predictor of postoperative disease recurrence in esophageal cancer. *Ann Surg Oncol*. 2011; 18: 3362-3369.
13. Ishizuka M, Nagata H, Takagi K, Iwasaki Y, Kubota K. Combination of platelet count and neutrophil to lymphocyte ratio is a useful predictor of postoperative survival in patients with colorectal cancer. *Br J Cancer*. 2013; 109: 401-407.
14. Tamhane UU, Aneja S, Montgomery D, Rogers EK, Eagle KA, Gurm HS. Association between admission neutrophil to lymphocyte ratio and outcomes in patients with acute coronary syndrome. *Am J Cardiol*. 2008; 102: 653-657.
15. Kantola T, Klintrup K, Vayrynen JP, Vornanen J, Bloigu R, Karhu T, et al. Stage-dependent alterations of the serum cytokine pattern in colorectal carcinoma. *Br J Cancer*. 2012; 107: 1729-1736.
16. Motomura T, Shirabe K, Mano Y, Muto J, Toshima T, Umemoto Y, et al. Neutrophil-lymphocyte ratio reflects hepatocellular carcinoma recurrence after liver transplantation via inflammatory microenvironment. *J Hepatol*. 2013; 58: 58-64.
17. Vincenzo Neri, Antonio Ambrosi, Giuseppe Di Lauro, Alberto Fersini, Tiziano Pio Valentino. Difficult Cholecystectomies: Validity of the Laparoscopic Approach. *JSLs*. 2003; 7: 329-333.
18. Michael Sugrue. Grading operative findings at laparoscopic cholecystectomy- a new scoring system. *World J of emergency surgery*. 2015; 10: 14.
19. Vivek MA, Augustine AJ, Rao R. A comprehensive predictive scoring method for difficult laparoscopic cholecystectomy. *J Minim Access Surg*. 2014; 10: 62-67.
20. Patel K, Wagstaff A, Mirza S. Department of Surgery, Ratio of neutrophil to lymphocyte count as a predictor of length of stay in acute cholecystitis.
21. <https://www.nice.org.uk/guidance/cg188/resources/costing-statement-193298365>.
22. Stephan Lorenz, Aishling Jaques, Stuart Andrews, Saj Wajed. Evidence to predict increased difficulty and adverse outcome for elective cholecystectomy. *International journal of surgery*. 2011; 9: 507.
23. Carruthers R, Tho LM, Brown J, Kakumanu S, McCartney E, McDonald AC. Systemic inflammatory response is a predictor of outcome in patients undergoing preoperative chemoradiation for locally advanced rectal cancer. *Colorectal Dis*. 2012; 14: e701-e707.
24. Cedres S, Torrejon D, Martinez A, Martinez P, Navarro A, Zamora E, et al. Neutrophil to lymphocyte ratio (NLR) as an indicator of poor prognosis in stage IV non-small cell lung cancer. *Colorectal Dis*. 2012; 14: 864-869.
25. Chua TC, Chong CH, Liauw W, Zhao J, Morris DL. Inflammatory markers in blood and serum tumor markers predict survival in patients with epithelial appendiceal neoplasms undergoing surgical cytoreduction and intraperitoneal chemotherapy. *Ann Surg*. 2012; 256: 342-349.
26. Hirota M, Takada T, Kawarada Y, Nimura Y, Miura F, Hirata K, et al. Diagnostic criteria and severity assessment of acute cholecystitis: Tokyo Guidelines. *J Hepatobiliary Pancreat Surg*. 2007; 14: 78-82.

Cite this article

Ahmed SE, Rehman S, Edilbe M, Jonker L, Canelo R (2017) Can Neutrophil-Lymphocyte Ratio Predict Operators' Difficulty in Early Cholecystectomies: A Retrospective Cohort Study. *Ann Emerg Surg* 2(3): 1016.